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ORIGINAL RESEARCH

Hydrophobic modification of bacterial cellulose using oxygen plasma treatment and chemical vapor deposition

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Abstract A new strategy for the surface modification of bacterial cellulose (BC) through the combination of oxygen plasma deposition and silanization with trichloromethyl silane (TCMS) is described. The combined use of the two techniques modifies both the surface roughness and energy and therefore maximizes the obtained hydrophobic effect. These modified membranes were characterized by Scanning Electron Microscopy (SEM), water contact angle measurements, Fourier-transform infrared spectroscopy (FTIR-ATR) and X-ray photoelectron spectroscopy (XPS), and its cytotoxic potential was investigated using both indirect and direct contact in vitro studies. The obtained results suggest an effective conjugation of TCMS to the surface of BC, leading to a highly hydrophobic surface, with a water contact angle of approximately 130°. It is also demonstrated that this is a stable and durable surface modification strategy, since BC remained hydrophobic even after 6 months, in dry conditions or after being submerged in distilled water for about a month. Importantly, this surface modification revealed no short-term cytotoxic effects on L929 and hDNFs cells. Altogether, these data indicate the successful development of a surface modification method that can be applied to BC, enabling the production of a biodegradable and hydrophobic platform that can be applied to different areas of research and industry.

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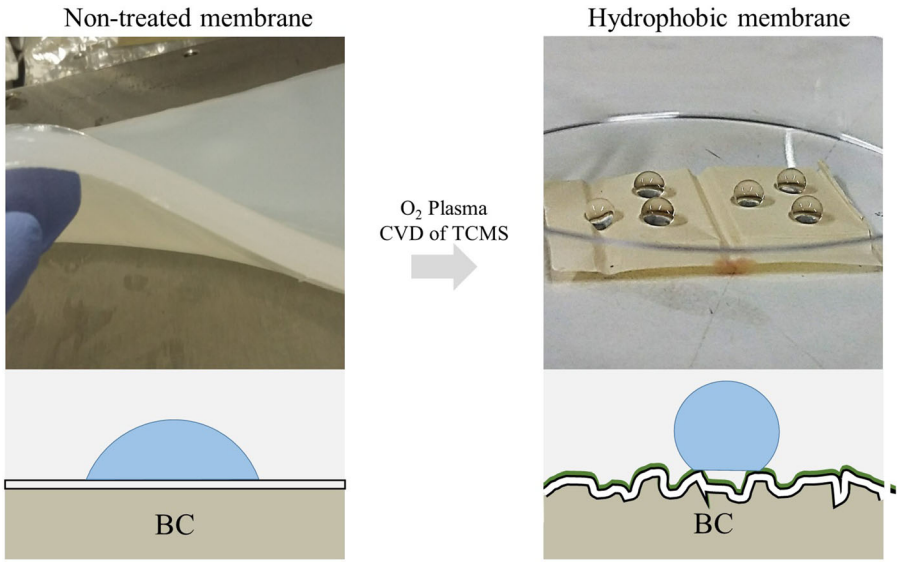
Graphic abstract



Keywords Bacterial cellulose · Chemical vapor deposition · Trichloromethylsilane · Oxygen plasma · Hydrophobic · Surface modification

Introduction

Cellulose is a highly abundant biopolymer, which is present mainly in plants (Klemm et al. 2005). Cellulose obtained from some bacteria, bacterial cellulose (BC), consists of a translucent and gelatinous film, produced by oxidative fermentation. Bacteria from the genera Komagataeibacter are the most efficient cellulose producers (Jedrzejczak-Krzepkowska et al. 2016). BC consists of a ultrafine network of well-arranged long cellulose microfibrils composed of (1 → 4)-D-anhydroglucopyranose chains bounded through β-glyosidic linkages and reinforced by an inter and intramolecular hydrogen-bonding network (Picheth et al. 2017). BC presents many advantages with regards to the vegetal counterpart, given its purity (Petersen and Gatenholm 2011; Picheth et al. 2017), mechanical strength (Shao et al. 2017), elasticity, and biocompatibility (Costa et al. 2017).

Depending on the fermentation process and conditions used, the shape of BC can be manipulated to obtain different forms, size, thickness and crystallinity, making BC adaptable to diverse applications (Picheth et al. 2017).

Taking advantage of BC natural properties, BC has been explored in the biomedical field, as a wound dressing material (Huang et al. 2014), as an anticoagulant agent (Fink et al. 2010) and as a scaffold for bone regeneration (Zimmermann et al. 2011), among other applications. In recent years, the relevance of BC increased in several fields of study as a flexible matrix to develop nanocomposites and materials with tailored properties for electronic or magnetic applications (Cacicedo et al. 2016). Although the use of BC has extended to different fields, the high hydrophilicity of this biopolymer (although desirable in many instances) can limit its range of applications. Therefore, a surface modification method that can modify the surface chemistry of BC would be beneficial to fully exploit the applicability of this outstanding biopolymer. Hydrophobization of BC sets grounds for several other applications including printing, food packaging and storage, microfluidic and bioassay devices (Song and Rojas 2013).

The wettability of a surface depends on its surface energy and roughness (Cassie and Baxter 1944; Wenzel 1936) and, therefore, different methods have been investigated to tailor materials hydrophobic characteristics. These strategies include the use of fluorocarbons, silicones and organic or inorganic materials, which by grafting, adsorption or chemical-

vapor deposition (CVD) have been applied to cellulose and decrease surface energy (Song and Rojas 2013). To obtain a stronger hydrophobic effect, methods which increase surface roughness can also be used prior to the surface energy reduction treatment, and these strategies include the use of electrospinning or electrospraying, nanoparticle deposition and plasma treatment (Calvimontes et al. 2011; Song and Rojas 2013).

Due to the high content of hydroxyl groups at the surface of BC, the reaction with fluorinated or alkylchlorosilylating agents can readily occur in the gas–solid interface, using CVD. Using this strategy, the surfaces are exposed to the chemical in its vapor phase and the hydrophobic molecules are grafted onto the BC surface (Fadeev and McCarthy 2000). This concept has been already employed for the modification of paper's surface chemistry (Glavan et al. 2013). Since BC presents a higher mechanical strength and purity relative to paper, surface modification of this biopolymer can enable the production of materials with a wide range of possible applications, including as a platform for the design of microfluidic devices (Costa et al. 2014). Therefore, the aim of this work is to establish a strategy to successfully modify BC surface, by combination of two methodologies, namely oxygen plasma deposition, which increases the roughness of BC, and silanization using trichloromethyl silane (TCMS) to decrease the surface energy. Using this strategy, it is hypothesize to obtain a biocompatible and long-lasting hydrophobic biomaterial that can resist wettability and therefore, unveil a large range of applications.

Materials and methods

Bacterial cellulose processing

Bacterial cellulose membranes (HTK CO., LTD, Vietnam) with an initial thickness of 12–15 mm were purified by alkali treatment with NaOH 0.1 M for 3 days (solution renewed every 12 h), under gentle stirring and at room temperature (RT). Afterwards, membranes were exhaustively washed with distilled water until the washing solution reached the pH of distilled water. Some of the membranes were also subject to washing with 5% (w/v) of sodium dodecyl sulphate (SDS; Sigma-Aldrich, St. Louis, MO, USA), for 5 days (solution renewed everyday), followed by extensive washing with distilled water, under agitation (100 rpm). This step was introduced only for the membranes used in the direct contact cell viability assay to further purify membranes by removing the remaining endotoxins and to avoid any impact on cell behavior (Leitao et al. 2016). After the washing steps, membranes were then sterilized by autoclaving at 121 °C, 1 bar, for 20 min, prior to storage.

Bacterial cellulose solvent exchange and densification

The BC membranes were processed by solvent exchange with ethanol, in order to accelerate drying, since this process allows the mechanical integrity of the membrane to be better preserved and yields a smother surface (Thuo et al. 2014). Firstly, membranes were compressed between two aluminum plates for 30 min, expelling the water entrapped in the BC network, until the thickness of the membrane was reduced by around 80%; then the membranes were immersed in increasing concentrations of ethanol solutions (25%, 50%, 75% and 100% v/v), for around 2 h in each stage, and stored in absolute ethanol until further use. When needed, BC membranes were again compressed between aluminum plates for 30 min (final thickness of approximately 2.3–2.4 mm). The densified membranes were allowed to dry at 37 °C for 24 h. The obtained dried membranes are referred to as "Non-treated".

Oxygen plasma treatment of BC membranes

In this study, oxygen plasma treatment was performed to increase the roughness of the BC membranes (Calvimontes et al. 2011). O_2 Plasma treatment was performed using a radio frequency (40 MHz) plasma reactor (Zepto Diener Electronics). Samples were exposed to O_2 plasma at a potency of 100 W, for 15 min. During the treatment, the gas flow was adjusted in order to keep a constant pressure of 100 Pa inside the reactor.

Chemical vapour deposition of trichloromethylsilane (TCMS)

Silanization was conducted in a reduced pressure chamber at a temperature set at 95 °C. The silanizing

reagent, TCMS (86 mM solution in anhydrous toluene), was placed inside the chamber together with the plasma-treated BC membranes and set to react for 60 min, at − 50 kPa. Following that period, the beaker containing the remaining TCMS (in toluene) was removed from the oven and replaced with only toluene which was left for 10 min for the washing of some of unbound TCMS upon toluene condensation on BC surface. After this, the membranes in the chamber, were subject to vacuum (− 50 kPa) for 5 min to further remove the toluene and obtain the dried BC membranes.

Fourier transform infrared: attenuated total reflectance (FTIR-ATR)

The chemical modifications were investigated by FTIR-ATR. FTIR-ATR spectra collected at room temperature over a scanning range of 600–4000 cm^{-1} with a resolution of 4.0 cm^{-1}, using a JASCO FT/IR 4100—Fourier Transform Infrared Spectrometer.

X-ray photoelectron spectroscopy (XPS)

The XPS analysis was performed using a Kratos AXIS Ultra HSA with VISION software for data acquisition and CASAXPS software for analysis. For analysis, an achromatic Al Kα X-ray source operating at 15 kV (90 W) was used, and the spectrometer was operated in FAT mode with 40 eV pass energy for ROI regions and 80 eV for survey. Data acquisition was performed at a pressure below 1×10^{-6} Pa.

Scanning electron microscopy (SEM)

The surface morphology of the membranes was analysed using a FEG-SEM (Ultra-high resolution Field Emisson Gun Scanning Electron Microscopy), NOVA 200 Nano SEM (FEI Company), scanning electron microscope (SEM). All specimens were pre-coated with a conductive layer of Au/Pd.

Profilometry

The surface roughness was analyzed using profilometry. The profiler (Ambios XP-Plus 200 Stylus) with an analysis speed of 0.1 mm/s and a stylus force of 1.0 mg was used to scan 1 mm in the surface of each sample.

Wettability studies: static and dynamic water contact angles (CA)

To obtain the static and dynamic water CA and characterize the surface wettability, the DataPhysics OCA 15 Plus set up was used. The system is composed of a syringe placed vertically, coupled to an automated dispenser while a digital image acquisition system records the droplet's lateral profile. Throughout the study, 3 μL of ultrapure water droplets were used as reference. The static CA analysis was performed with the embedded software (SCA 20) using the Laplace-Young approximation model with at least three measurements per sample. The stability of the surface properties was investigated by measuring the static water CA, in properly stored samples, sealed in dry vessels at room temperature, for up to 6 months. For further evaluation of the stability and durability of the surface modification of BC, the samples were also submerged in distilled water, for up to 28 days at room temperature. After drying, the static contact angles were measured once more.

The dynamic water CAs, namely the advancing and receding CA, were measured by changing the volume of the droplet. In short, 3 μL droplet was first placed on the surface and the volume of the droplet was gradually increased, while recording the advancing contact angle. The receding angle was measured in the same way, but in this case gradually decreasing the droplet volume. The difference between the advancing and receding contact angles gives information on the surface hysteresis. To demonstrate the high water adhesiveness of the BC-treated surface, photographs of the water droplet on BC surface were taken with a tilting angle of 0, 90 and 180° (DataPhysics OCA 15 Plus).

Membrane's processing for in vitro assays

In the mentioned cases, a washing step was performed, either after obtaining the NT or the PlasSil-treated BC, to decrease the cytotoxic potential. This was achieved by submerging the BC membranes in MilliQ water (1 mL/cm^2) for 24 h in agitation at 60 rpm, after which the membranes were allowed to dry overnight at room temperature. Washed and non-washed membranes were sterilized using ultraviolet (UV) light before the in vitro viability assays, 30 min/side. This procedure is described as acceptable for the removal of

common pathogenic bacteria, being easy to perform and with no residual effects left on the surface (Katara et al. 2008).

Cell culture conditions

L929 immortalized mouse lung fibroblasts cell line was purchased from ATCC. L929 cells were routinely cultured in Eagle's Minimum Essential Medium (EMEM), supplemented with 10% horse serum (HS; both from ATCC) and 1% penicillin:streptomycin (P/S; Merk, Darmstadt, Germany). Subcultures were performed by trypsinization and cells were used until passage 10. For the assays, L929 cells were seeded in 96-well plates, at a density of 0.5×10^5 cells/mL. To allow cell adhesion, the plates were incubated overnight in humidified atmosphere of 5% CO_2 at 37 °C, before any treatment.

The hDNFs (human dermal neonatal fibroblasts; ZenBio) primary cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% P/S (Biowest) and 1% amphotericin B (Capricorn Scientific) and kept at 37 °C in a humidified atmosphere of 5% CO_2, and used until passage 8. For cytotoxicity assays, hDNFs were seeded in 24-well plates and incubated in 500 µL of complete DMEM and allowed to reach approximately 80-90% confluence.

In vitro cytotoxicity evaluation: indirect and direct contact assays

The effect of the surface treatment of BC membranes on cell viability was assessed according to ISO 10993-5 (2009) standard, through indirect and direct contact viability assays.

For the indirect contact experiment, the cytotoxicity of leaching compounds from the membranes on the cellular metabolism was investigated using L929 cells. Extracts were obtained by incubating the sterilized membranes in sterile complete culture media (3 cm^2/mL) for 24 h, at 37 °C and agitation at 200 rpm. In addition to evaluating the effect from whole extracts (100%) on cell viability, 50% extracts were also prepared by dilution in fresh culture media. Complete culture media subject to the same conditions, but with no membranes, was used as blank. Cell culture media with and without 30% DMSO were used as positive and negative control, respectively. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24 h of incubation with extracts. Briefly, at the end of the experiment, culture media was removed and 50 µL of 1 mg/mL MTT solution (in phenol Red free media) was added in each well and incubated for 2 h at 37 °C, protected from light. In metabolically active cells, the tetrazolium compound is reduced forming a colored insoluble formazan product, which was solubilized with isopropanol (100 µL/well), with color intensity being proportional to the number of viable cells (Mosmann 1983). Absorption was determined spectrophotometrically at 570 nm using a microplate reader spectrophotometer (Bioteck Plate Reader, Synergy MX) and cell viability expressed as percentage (%) relative to a non-treated control.

For the direct contact viability assay, hDNFs primary cells were used. Samples (approximately 1 cm^2 with less than 10% of weight variation) were directly put into the wells containing fibroblasts and the medium was refreshed, allowing the simultaneous test of the material itself and the leachable substances thereof. All experiments were carried out in triplicate and cells that were not in contact with the materials were used as control. The resazurin-based assay was used to assess cytotoxicity after 24 h incubation. Similar to the MTT reduction assay, the resazurin-based assay is able to monitor the cell's redox state, in which the active metabolism of viable cells can reduce resazurin into the pink and fluorescent product resorufin (Riss et al. 2016). Briefly, culture medium was removed and replaced with complete medium with 20% (v/v) resazurin (Sigma-Aldrich, Germany) and incubated at 37 °C in a humidified incubator with 5% CO_2 for 2 h. Afterwards, 3 x 100 µL per well were transferred to a 96-well black plate/clear bottom and fluorescence was measured (λex = 530 nm/λem = 590 nm) using the aforementioned microplate reader spectrophotometer.

Manufacturing of a BC-based platform

A preliminary study was performed to address one of the possible applications of the hydrophobically modified BC. Hypothesizing the use of BC membranes as a platform for in vitro cell culture, BC was shaped upon drying, embossing the solvent-exchanged membrane between two complementary molds, which

would imprint its shape on BC. Molds were designed using the Fusion 360 software and the 3D printer Ultimaker 2 + to obtain the Polylactic Acid (PLA) thermoplastic moulds. These molds were intended to obtain the features of wells and channels which would enable the physical receptacle of cells, and the continuous feeding of cell culture media, and respective removal of excreted by-products. BC within the molds was dried at 37 °C, for 40 h to complete the process and obtain the 3D membrane that was later subject to the surface modification treatments previously described.

Statistical analysis

The statistical analysis in this study was performed using the Graphpad Prism 6 software. For the cell viability studies, experiments were performed in triplicate, in three different independent assays and significant differences between the different treatments on cell viability were identified using unpaired *t* test (2 tailed) for the indirect and direct contact viability assays.

Results and discussion

Infrared vibration spectra

In order to characterize the chemistry of the modified surface, Attenuated Total Reflection Fourier Transform infrared spectroscopy (FTIR-ATR) analysis was used. Figure 1 shows the FTIR-ATR spectra with the characteristic bands of native BC, namely O–H stretching at 3345 cm^{-1}, C-H stretching at 2900 cm^{-1}, CH_2 bending at 1420 cm^{-1}, and C–O–C skeletal vibrations of pyranose ring at 1055 cm^{-1}, as previously reported by Kumar et al. (2014). The peak assigned to Si-CH_3 at 1280 cm^{-1} (Mohd et al. 2016) indicates the presence of TCMS at the surface of BC. Bands for Si–O–Si and -Si–O–C bond at 1160 cm^{-1} and 1108 cm^{-1}, respectively (Rachini et al. 2012), overlapped with band C–O–C corresponding to the cellulose skeletal vibration in the range 970–1250 cm^{-1}, however these are slightly higher in the case of Sil and PlasSil samples.

Surface chemical modifications

XPS analysis was used to characterize the relative atomic concentration of carbon (C), oxygen (O) and silicon (Si) on the BC membranes obtained using the different surface modification treatments. The results from the XPS analysis are shown in Table 1. The non-treated and plasma-treated membranes reveal a similar C and O elemental profile. An increase in Si corresponds to the silanization treatment whether there was a pre-treatment with O_2 plasma or not, the amount of Si (%) being similar, which suggests that the efficiency in the conjugation of TCMS to BC does not depend on the O_2 plasma pre-treatment. Therefore, since the deposition of TCMS appears to be similar in both treatments, the increased hydrophobicity obtained in the PlasSil treatment is explained by the increased roughness conferred by the O_2 plasma pre-treatment (Seo et al. 2015). As described by Wenzel (1936) the increased roughness contributes to higher hydrophilicity in a hydrophilic surface and to a higher hydrophobicity in a hydrophobic one. As expected, the increase in Si (%) is accompanied by a decrease in the relative percentage of C and O.

Morphological features

With the aim of increasing the BC surface hydrophobicity, samples were allowed to react with trichloromethyl silane (TCMS), after pre-treatment with O_2 plasma. To characterize surface morphology of the produced BC membranes, samples were analysed by SEM. Figure 2 presents the scanning electron micrographs of the membranes at different stages of the process.

Scanning electron micrographs of the surface of non-treated and silanized samples without oxygen plasma pre-treatment revealed in general a smooth surface, with a well-defined and closed fibre network, without pores (Fig. 2a, c). The SEM analysis of the plasma-treated fibers, followed or not by silanization, revealed an increased roughness, open porosity and no discernible fiber network (Fig. 2b, d). This remarkable alteration in the BC membrane surface morphology is induced by the oxygen plasma treatment. Alteration in the roughness of cellulose, induced by oxygen plasma is, according to Calvimontes et al. (2011), caused by the decomposition of polymer chains and oxidation reactions.

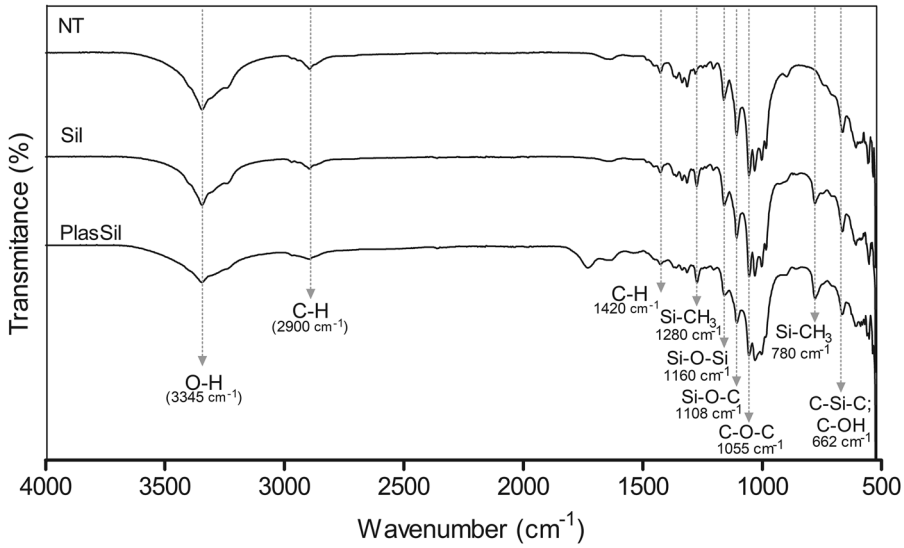


Fig. 1 FTIR spectra of BC membranes subject to different surface treatments (NT—Non-treated; Plas—O_2 Plasma-treated; Sil—Silanized BC without plasma treatment; PlasSil—Silanized BC with O_2 plasma pre-treatment

Table 1 BC membrane surface elemental composition obtained by XPS for Non-treated (NT) samples, O_2 plasma-treated membranes (Plas), silanized membrane without plasma treatment (Sil) and silanized membrane with O_2 plasma pre-treatment (PlasSil)

At (%)				
Element	NT	Plas	Sil	PlasSil
C 1s	55.6	54.8	51.9	51.3
N 1s	–	0.8	–	0.5
O 1s	44.4	44.4	43.4	43.5
Si 2p	–	–	4.8	4.7

The internal structure of BC was also analyzed using SEM, and the results shown in Fig. 3 demonstrate that the increased roughness induced by oxygen plasma treatment is confined to the surface and that the overall lamellar structure of the membranes is preserved.

Surface profilometry

To further characterize changes in the topographic roughness of BC, samples were also analyzed using optical profilometry. With this analysis, the grooves and ridges of a surface are screened and the thickness, or distance of each point to the reference level, can be plotted. From the results shown in Fig. 4 it is noticeable that the non-treated and Sil-treated BC membranes present a similar and smoother roughness profile when compared with the PlasSil samples. PlasSil membranes showed a high variation of thickness throughout the analyzed area, which is indicative of a high roughness and higher surface area. These results are in agreement with the observed using SEM, and indicate that the O_2 plasma treatment is essential for the alteration of surface roughness. When aiming at obtaining a hydrophobic surface, increasing surface roughness prior to the deposition of a hydrophobic coating leads to an increased hydrophobicity (Wenzel 1936). In this sense, it is expected that this treatment should improve the obtained hydrophobic effect.

Wettability studies

To analyze the effectiveness of the surface wettability modification method described in this work, the static and dynamic contact angles (CA) were measured. The static water CAs for the BC membranes with different surface modification are presented in Table 2.

The effect of the O_2 plasma treatment is complex. On the one hand, it is likely to change the surface chemistry (nitrogen is detected in the plasma treated samples by XPS); on the other hand, the increased roughness associated to the low contact angle will generate capillary effects, since a concave meniscus

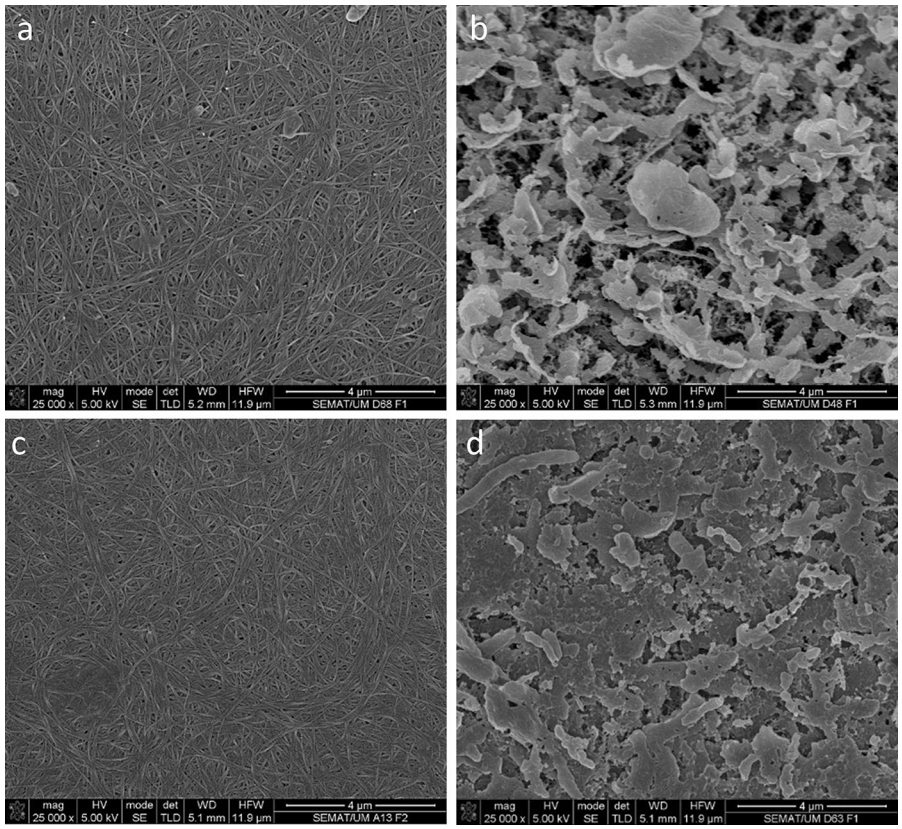


Fig. 2 SEM micrographs of the produced BC membranes. **a** Non-treated membrane, **b** O_2 plasma-treated membrane, **c** silanized membrane without O_2 plasma treatment and **d** silanized membrane with O_2 plasma pre-treatment (mag 25000 ×, scale bar = 4 μm)

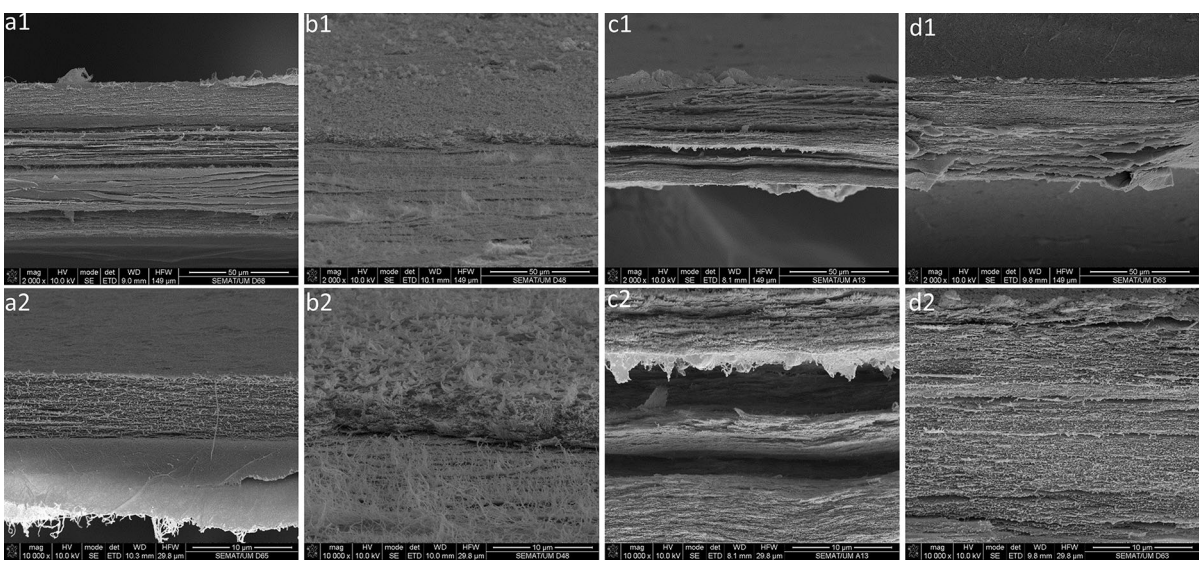


Fig. 3 SEM micrographs of the transversal section of the BC membranes. **a** Non-treated membrane, **b** O_2 plasma-treated membrane, **c** silanized membrane without plasma treatment, **d** silanized membrane with O_2 plasma pre-treatment (1, scale bar = 50 μm; 2, scale bar = 10 μm)

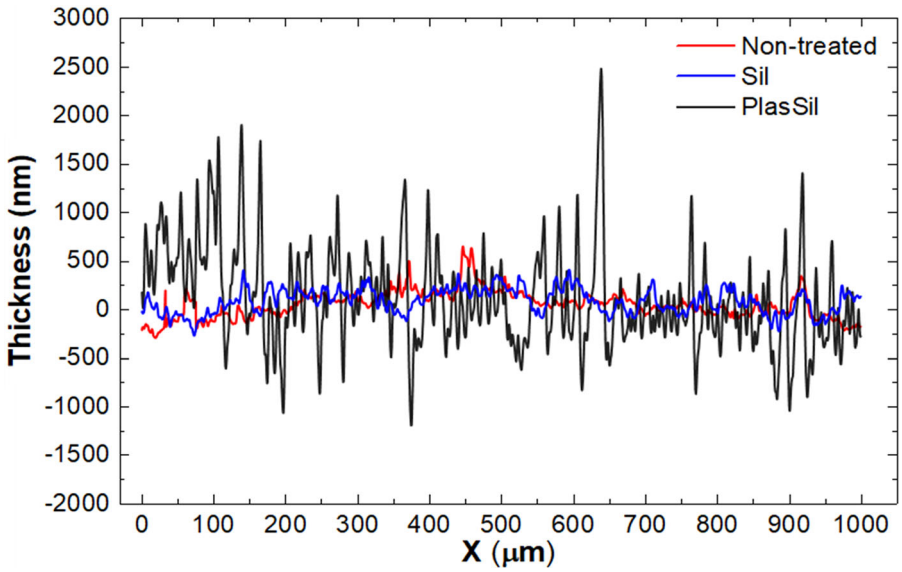


Fig. 4 Profilometry analysis of the non-treated BC membranes and the samples subject to silanization without plasma treatment (Sil) and silanization with O_2 plasma pre-treatment

Table 2 Static water contact angles obtained for the different surface modification treatments of the BC membranes

BC sample		Initial CA
NT		24.9 ± 1.9
Plas		26.6 ± 1.2
Sil		119.8 ± 2.3
PlasSil		132.6 ± 7.2
Conditions Stability assay (PlasSil)	Time (days)	Final CA
Dry	180	135.4 ± 1.2
Wet	7	110.3 ± 14.5
	28	108.2 ± 6.5

NT—Non-treated; Plas—O_2 Plasma-treated; Sil—Silanized BC without plasma treatment; PlasSil—Silanized BC with O_2 plasma pre-treatment

will arise, favoring wettability. However, the contact angle formed upon deposition of the water droplet (26.6°) does not significantly change, as compared with the non-treated material (24.9°). Differently, the treatment with TCMS on its own (Sil) is able to increase the static contact angle to 119.8°, due to the presence of the hydrophobic moieties of Si-CH_3, as detected by XPS and FTIR. Surface O_2 plasma treatment previous to the TCMS silanization (PlasSil) further increases the contact angle to 132.6°. The higher hydrophobicity is explained by the formation of a convex meniscus, due to high contact angle, leading to air entrapment in the rough surface, which contributes to a higher contact angle and lower wettability of the surface, a phenomena well documented in the literature (Cassie and Baxter 1944).

Given the successful modification of BC wettability using the PlasSil treatment (O_2 plasma followed by silanization), the stability of the surface modification obtained after this method was investigated over time, under different conditions. Samples stored at room temperature were investigated by measuring the static CA after 6 months (180 days). The results shown in Table 2 demonstrate that the surface properties of the dry samples remain stable, with no significant changes in the CA over time.

The stability of the PlasSil surface modification was moreover investigated by submerging the membranes in distilled water up to 28 days at room temperature. This assay was essential to understand if the conjugation of TCMS to BC was effective and stable. The results reveal a significant decrease of the CA in the first 7 days of contact with distilled water, with a stabilization at a value around 110° (Table 2). The decreased CA values together with the higher standard deviation suggests some leaching of the surface coating and a higher heterogeneity in the samples.

Nevertheless the CA remained always high, the BC surface continuing highly hydrophobic (Song and Rojas 2013) even after 28 days submerged in water.

In rough surfaces, wettability should not be characterized just by static CA measurements. In fact, the droplets can present multiple equilibrium positions, and therefore there is a maximum CA value, known as advancing CA (θA) and a minimum CA value, which is called receding CA (θR), with the difference between the two values being called CA hysteresis (Bhushan and Nosonovsky 2010). The results represented in Table 3 show that both surface modification strategies, Sil or PlasSil, result in a similar CA hysteresis around 85°. This high CA hysteresis is the result from BC surface roughness as well as from some chemical heterogeneity. Whereas the results shown in Fig. 2 show a remarkably different surface roughness after the Sil and PlasSil surface treatments, a similar CA hysteresis is obtained (Table 3).

The large difference between advancing and receding CAs may translate a high water adhesiveness, as can indeed be observed in this case, the water drop remaining adhered to BC surface even when the sample is rotated by 90 and 180° (Fig. 5b, c). Water repellency associated with a low CA hysteresis means a very low roll-off angle, which is desirable in the engineering of self-cleaning microchannels for liquid flow applications, for example. This wetting regime is called the "Lotus effect" (Bhushan and Jung 2011). Although not intuitive, a hydrophobic surface can also display high water adhesion. When water penetrates into the micro-roughness, but not the nano-roughness, of a solid surface, another wetting regime exists which is known as the "petal effect", characterized by superhydrophobicity associated with a high water adhesion (Bhushan and Nosonovsky 2010). Superhydrophobic surfaces with high water adhesion have potential for application in the transport of small liquid volumes, such as in open microfluidic devices (Hong et al. 2007). As previously mentioned, the advancing CA represents the maximum value for static CA in a heterogeneous and rough surface, however in the results obtained, a lower value for the advancing CA relative to de static CA is apparent which can be attributed to the sample's heterogeneity.

Table 3 Contact Angle (CA) hysteresis obtained from the advancing (θA) and receding water CA (θR) for the different surface modification treatments of BC: Sil—Silanized BC without plasma treatment; PlasSil—Silanized BC with O_2 plasma pre-treatment

	θA	θR	CA hysteresis (°)
Sil	110 ± 1	26 ± 3	84
PlasSil	122 ± 5	36 ± 4	86

In vitro toxicity studies

To further investigate the applicability of this hydrophobically modified substrate, its effects on cell viability were investigated. An indirect contact assay was performed in which L929 cells were exposed to culture media containing the extracted leachable components from the PlasSil-modified or NT BC surface. The L929 cell line was used to screen the cytotoxicity of the developed membranes, since this is a well-established cell model (Faria et al. 2009). As depicted in Fig. 6, after 24 h in contact with the whole extracts (100%) of unwashed NT membranes, there was a significant and unexpected reduction in cell viability. This cytotoxic effect was even more pronounced than in the unwashed PlasSil membranes. Given the purity of BC, and its demonstrated biocompatibility in vitro (Silva et al. 2018) as well as in vivo (Helenius et al. 2006), with good applicability as a scaffold for tissue engineering (Svensson et al. 2005), this cytotoxic effect of the NT membranes was initially attributed to insufficient washing of BC membranes. However, the deleterious effect from NT membranes on cell viability was later found to be associated with ethanol residues from the solvent-exchange stage. Sterilization using 70% (v/v) ethanol was exploited for the decontamination of the membranes used in the in vitro assays and, in all tested conditions, this resulted in a complete inhibition of cell viability, when compared to the effect from non-sterilized samples (results not shown). Therefore, this method was discontinued and UV radiation was used for membrane sterilization, with no visual signs of contamination throughout the assay.

Nonetheless, the solvent-exchange step was maintained, therefore in order to decrease the cytotoxic effects from ethanol and TCMS leachable components on cell viability, a washing step using MilliQ water was introduced before in vitro experiments in both NT and PlasSil-treated BC membranes. This resulted in a

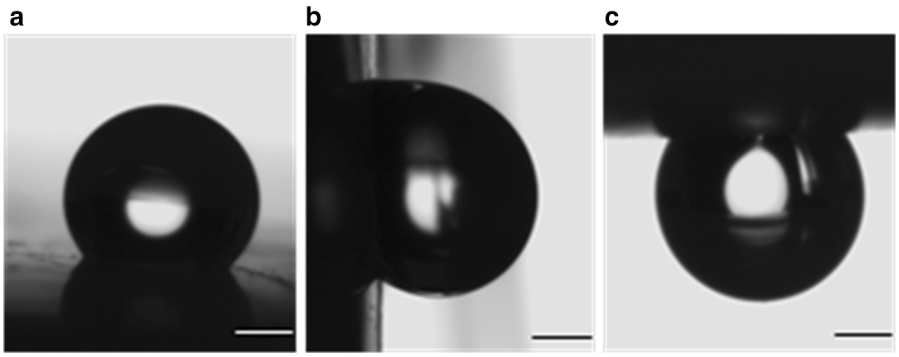


Fig. 5 Shape of droplets (MiliQ water) on the surface of PlasSil-modified BC at different tilting positions, at 0° (**a**), 90° (**b**) and 180° (**c**) (Scale bar = 0.5 mm)

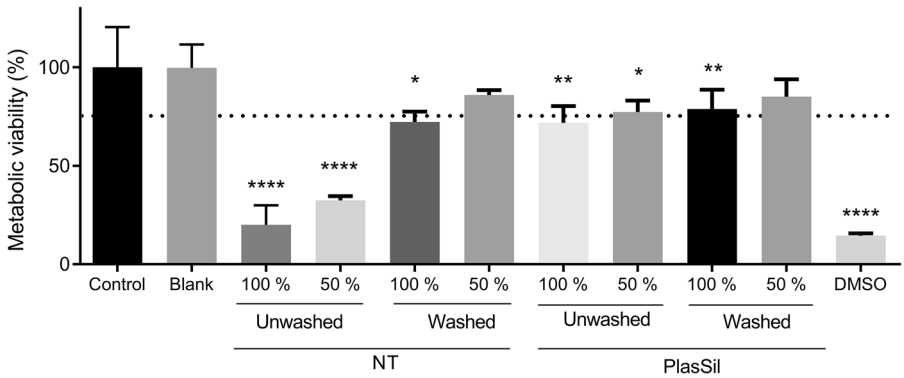


Fig. 6 Effects from the extracts of the hydrophobically modified BC on cell viability. L929 cells were incubated with the whole (100%) or diluted (50%) cell culture extracts, from either washed or unwashed non-treated (NT) or PlasSil-treated BC membranes. Cell viability was evaluated using the MTT reduction assay after 24 h. Cell viability is expressed in % relative to the non-treated control (*$p \leq 0.05$; ** $p \leq 0.001$)

significant improvement of cell viability in response to the diluted and whole extracts from NT membranes (Fig. 6). The fact that the unwashed PlasSil membranes, which could have leachable residues of TCMS and toluene, showed a lower cytotoxic effect than the unwashed NT membranes also corroborates the hypothesis that the presence of ethanol residues primarily accounts for the observed cytotoxic effects. Since the reaction with TCMS occurs at 95 °C, for 1 h, this could allow a more efficient evaporation of ethanol residues. Therefore, washing after the PlasSil surface modification resulted a lower degree of improvement of cell viability. The whole extracts from washed PlasSil membranes caused a significant reduction on cell viability, noteworthy cell viability in these cases remained above 70%, which can be considered as non-cytotoxic (ISO 10993-5 2009). As expected, the 50% extract had a lower effect on cell viability, with an innocuous effect, from the analysis performed. These results suggest that the developed surface treatment for the hydrophobization of BC has no short-term effects on cell viability.

A direct contact study was also performed to clarify any cytotoxic potential. This time, a primary cell culture was used, namely hDNFs. The use of a primary cell culture of fibroblasts, which is more sensitive than routinely used cell lines, phenotypically closer to its in vivo counterparts (Pan et al. 2009) and in this case, from human origin, can help to detect more accurately any cytotoxic effects from the PlasSil treatment. In this study, NT and PlasSil-modified BC membranes were directly brought into contact with cells and, as can be seen from Fig. 7, after 24 h with the washed and unwashed PlasSil-modified BC membranes, the cell viability remains very close to the control with no substantial alterations. It can also be noted that both

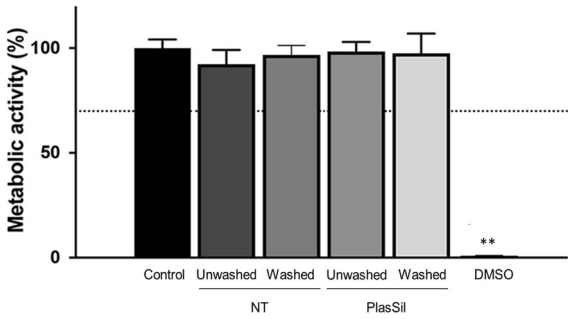


Fig. 7 Effects from the direct contact of hydrophobically modified BC membranes on cell viability. hDNFs primary cells were directly incubated with the non-treated (NT) or PlasSil-treated BC membranes. Cell viability was evaluated after 24 h by the resazurin-based assay and cell viability expressed in % relative to the control (**$p \leq 0.001$)

the unwashed and washed NT BC membranes showed no cytotoxic effect, contrary to what was observed in the indirect contact study. The extraction conditions of the indirect contact assay (high speed—200 rpm—and at 37 °C) attempt to simulate and exaggerate the leaching of the possible cytotoxic compounds present in a given material (ISO 10993-5 2009). Although the cell models used in the indirect and direct contact studies were different, in the indirect contact assay cells were exposed, from the beginning to the end of the experiment, to culture media containing the released compounds over a 24 h window, under the conditions needed to extract the majority, if not all, of the leaching compounds. On the other hand, in the direct contact study, cells are incubated in fresh media and the sample placed in the centre of the well containing the cells (ISO 10993-5 2009). This allows a simultaneous testing of direct contact toxicity and the leaching of compounds to media, however, leaching of any compounds is expected to be far more slow than it would be under agitation. Altogether, these results suggest that the direct contact of NT or PlasSil-treated BC membranes has an innocuous effect on cells, and that the leaching of compounds from BC can only be a concern in the case of intensified extraction conditions.

This is not the first report over the use of plasma treatment followed by silanization for cellulose surface modification. It was previously reported by Zhang et al. (2016) the use of Argon plasma and silanization by a physical-soaking method to obtain superhydrophobic surface of vegetable cellulose. Nevertheless this is, to our knowledge, the first report of the use of oxygen plasma followed by silanization using CVD. The use of CVD allows the easy, effective and reproducible surface modification, leaving impurities in solution out of the reaction and allowing the surface modification of shaped substrates (Creighton and Ho 2010).

Attempting to change the surface chemistry of paper, Glavan et al. (2013) proposed a simple and rapid method which consists in CVD of organosilanes, representing a technique able to modify vegetal cellulose to obtain surfaces with highly hydrophobic properties. In this case, we propose that the additional pre-treatment with oxygen plasma can enhance the hydrophobic effect obtained after silanization in BC, which is a more pure, resistant and biocompatible cellulose source. Given the reported effect of rough hydrophobic surfaces on improving cell adhesion (Wang et al. 2003), it is proposed that the PlasSil surface modification strategy is most suitable for the use of BC as a platform for cell culture.

The hydrophobic modification of cellulose described herein can also be subject to further modifications if needed. Namely, the concentration of TCMS can be increased, or the silanization duration can be prolonged to obtain higher CA measurements and a higher conjugation efficiency. Altogether, the results obtained regarding the exposure of cells to the PlasSil-treated BC membranes are very promising, showing a good biocompatibility in both direct and indirect contact experiments. Further studies are required, but the present one already shows the suitability of this biopolymer for applications as cell culture platform or in the design of microfluidic devices.

Manufacturing of a BC-based platform

Given the biocompatibility of the hydrophobic modification of BC herein shown and the current concern with plastic overuse (North and Halden 2013), the hypothesis that this biodegradable biomaterial could be used as a platform for the continuous cell culture was raised. Some preliminary studies were conducted in which the shape of BC was tailored to obtain the features of channels and wells, as represented in Fig. 8. This demonstrates the successful shaping of BC to obtain a platform which, due to the high water

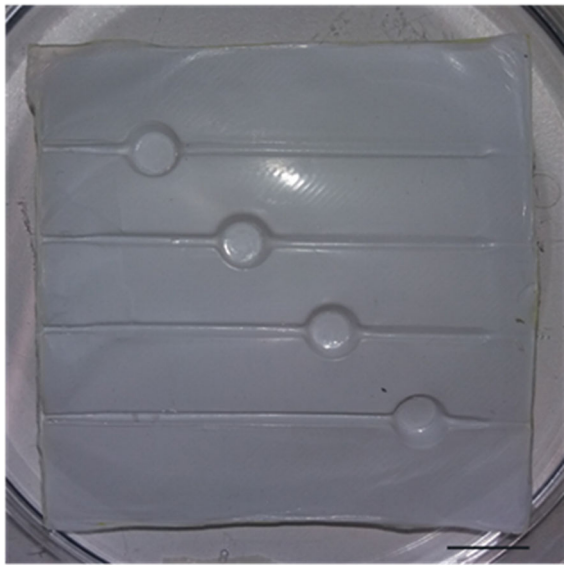


Fig. 8 BC platform obtained after embossing of BC between the two complementary molds. (Scale bar = 1 cm)

resistance and biocompatibility, can show a new array of applications.

Conclusions

In this work, a combination of two surface modification methods was used to allow the production of highly hydrophobic BC membranes. The use of oxygen plasma followed by silanization using TCMS allowed to obtain a higher roughness and lower surface energy, respectively. This resulted in a hydrophobic BC membrane that can withstand wet conditions for up to a month and has no short-term effects on cell viability, leading to the possibility of using this material as a platform for cell culture and microfluidic devices, replacing the use of common plastics.

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Compliance with ethical standard

Conflict of interest No potential conflict of interest was reported by the authors

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